

## Special Section on Drug Delivery Technologies—Minireview

# Protein Chimerization: A New Frontier for Engineering Protein Therapeutics with Improved Pharmacokinetics

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### ABSTRACT

With the advancement of medicine, the utility of protein therapeutics is increasing exponentially. However, a significant number of protein therapeutics suffer from grave limitations, which include their subpar pharmacokinetics. In this study, we have reviewed the emerging field of protein chimerization for improving the short circulatory half-life of protein therapeutics. We have discussed various aspects of protein therapeutics

aiming at their mechanism of clearance and various approaches used to increase their short circulatory half-life with principal focus on the concept of chimerization. Furthermore, we have comprehensively reviewed various components of chimera, such as half-life extension partners and linkers, their shortcomings, and prospective work to be undertaken for developing effective chimeric protein therapeutics.

### Introduction

With the advent of the 21st century and the growth of recombinant DNA technology, there has been significant progress in the field of biopharmaceuticals, most of which could be attributed to the development of protein therapeutics. In addition, the development of cloning methods and systems for recombinant protein production has certainly advanced the research and development of protein therapeutics (Gellissen, 2005). Recently, it has been reported that 129 distinct biopharmaceuticals have entered the market between January 2014 and July 2018 in the US and European Union regions (Walsh, 2018). The approval of Humulin in 1982 has been a pioneering landmark in journey of recombinant protein therapeutics (De Meyts, 2017). Some other major approvals that followed the approval of Humulin are Nutropin (somatotropin) in 1985 and Alteplase (tissue plasminogen activator) in 1987, followed by Epogen (erythropoietin) and Neupogen (granulocyte colony-stimulating factor) in 1989 and 1991, respectively (De Meyts, 2017). Since the achievements of these milestones, protein therapeutics have come a long way and have proved to be extremely effective for treatment of various diseases

(Leader et al., 2008; Walsh, 2014, 2018; Lagassé et al., 2017). It is estimated that at the global level, the share of biopharmaceuticals entities among clinically studied pharmaceutical products is about 40 percent (Walsh, 2018).

In comparison with the small-molecule drugs, the use of protein therapeutics offers substantial advantages, which include lower propensity of adverse events, better tolerance, and the wide-ranging application of protein therapeutics as a replacement therapy for a variety of disorders (Leader et al., 2008; Walsh, 2010, 2014, 2018). In addition, because protein therapeutics offer shorter timeline of approval from various drug regulatory agencies and better patent protection, this makes them commercially lucrative for the pharmaceutical enterprises (Leader et al., 2008).

However, the application of protein therapeutics still suffers from serious limitations that include poor pharmacokinetics and immunogenicity (De Groot and Scott, 2007; Strohl, 2015). Several mechanisms of protein metabolism can contribute to poor pharmacokinetics of protein therapeutics (Kontermann, 2011, 2016; Meibohm, 2012). In addition, immunogenicity of recombinant protein therapeutics is also one of the major challenges as it leads to development of neutralizing antibodies and undesirable immune responses in the patients, thus resulting in reduced efficacy, rapid excretion, and multiple adverse effects (De Groot and Scott, 2007; Purcell and Lockey, 2008; Baker et al., 2010; Chirmule et al., 2012; Kimchi-Sarfaty et al., 2017). In this review, we have particularly focused upon the use of chimerization approach to improve the pharmacokinetics of protein therapeutics.

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**ABBREVIATIONS:** CTP, C-terminal peptide; ELP, elastin-like peptide; Fab, fragment antigen binding; FcRn, neonatal Fc receptor; GLP-1, glucagon-like peptide; HAP, homo-amino-acid polymer; HLEP, half-life extension partner; IL, interleukin; PEG, polyethylene glycol; scFv, single-chain variable fragment; Tf, transferrin.

## Pharmacokinetics of Protein Therapeutics

Among various pharmacokinetic parameters, half-life, defined as the time required for the drug concentration to be reduced to one-half in the body, is of particular importance, as it is pivotal in determining the dosage frequency of the drug (Meibohm, 2012).

### Issues with Nonconjugated Protein Therapeutics

As mentioned above, several protein therapeutics show suboptimal pharmacokinetic attributes. For instance, exenatide, which is an incretin mimetic, has a half-life of ~2.4 hours. Similarly, glucagon-like peptide (GLP-1) has a half-life of up to 2 minutes because it is cleaved readily by dipeptidyl peptidase 4 (Bond, 2006; Diao and Meibohm, 2013; Strohl, 2015). The short circulatory half-life of the protein therapeutic may result in issues like frequent dosing and patient noncompliance, thereby making it important to address the metabolic mechanisms of protein therapeutics (Strohl, 2015; Lagassé et al., 2017).

### Mechanisms of Protein Metabolism and Clearance

A protein, whether endogenous or externally administered, goes through the process of absorption, distribution, metabolism, and elimination in the body (Strohl, 2015). In terms of delivery, oral route is the most convenient, patient compliant, and most preferred route for small-molecule drugs (Meibohm, 2012). Although, a recent study has demonstrated successful oral delivery of insulin, but owing to gastrointestinal degradation and issues with bioavailability, oral route is generally not suitable for delivery of therapeutic proteins (Meibohm, 2012; Tibbitts et al., 2016; Abramson et al., 2019). Interestingly, protein and peptide therapeutics have been delivered in the form of oral inhalations, as inhaled protein therapeutics with molecular weight of up to 40 kDa could achieve significantly high systemic bioavailability (de Kruijf and Ehrhardt, 2017). It is important to note that inhalable versions of insulin, marketed as Exubera and Afrezza, have been approved by the Food and Drug Administration (de Kruijf and Ehrhardt, 2017). In practice, the parenteral route is considered to be most suitable for administration of protein therapeutics (Meibohm, 2012). Hence, i.v., s.c., and i.m. routes are viable options for administration of protein therapeutics (Meibohm, 2012; Strohl, 2015).

After administration, the absorption of therapeutic proteins occurs mostly through the blood vessels and lymphatics (Supersaxo et al., 1990; Meibohm and Braeckman, 2007; Meibohm, 2012; Strohl, 2015). The distribution of proteins is mostly dependent upon their molecular weight, binding to other proteins in the plasma, their overall charge, and their extent of lipophilic nature; however, owing to their large size, their distribution is largely confined to the extracellular compartment (Meibohm, 2012; Diao and Meibohm, 2013; Strohl, 2015). The metabolism and elimination of proteins occur significantly by proteolytic mechanisms (Meibohm, 2012; Strohl, 2015). The renal route of elimination also contributes considerably to the metabolism of proteins and is mostly selective towards molecular size and charge (Meibohm, 2012; Strohl, 2015). Proteins with molecular weight less than 60–70 kDa are eliminated relatively swiftly through glomerular filtration, and the passage of negatively charged molecules is impeded in comparison with neutral or cationic molecules (Deen et al., 2001; Meibohm, 2012; Strohl, 2015; Tibbitts et al., 2016). There is also a substantial contribution of liver to the metabolism of proteins, and, depending on

their size, protein therapeutics may be taken up into the hepatocytes by passive diffusion or via uptake facilitated by carriers or receptors (Meibohm, 2012). The protein therapeutics also undergo receptor-mediated cellular uptake via the target receptor of the protein therapeutics expressed by any cell, by a process known as target-mediated drug disposition, which can be regarded as the stepping stone in the elimination of protein therapeutic (Tang et al., 2004; Mager, 2006; Meibohm, 2012). In addition, the neonatal Fc receptor (FcRn) plays a pivotal role in the disposition of IgGs and protein therapeutics fused with a fraction of Ig (e.g., Fc), or albumin, by a process called FcRn-mediated recycling (Fig. 1) (Kim et al., 2007; Roopenian and Akilesh, 2007; Wang et al., 2008; Kontermann, 2011, 2016; Meibohm, 2012; Schmidt, 2013b; Sockolosky and Szoka, 2015). It has been observed that FcRn-mediated recycling, owing to differential affinity of Fc region toward FcRn, is responsible for varying circulatory half-lives of IgG subtypes, with IgG1, IgG2, and IgG4 having substantially long half-lives (~18–21 days) in comparison with IgG3 (~7 days) (Kim et al., 2007; Dirks and Meibohm, 2010; Meibohm, 2012). Thus, various mechanisms of clearance can be targeted for improving the pharmacokinetics of therapeutic proteins.

## Strategies for Improving Pharmacokinetics of Protein Therapeutics

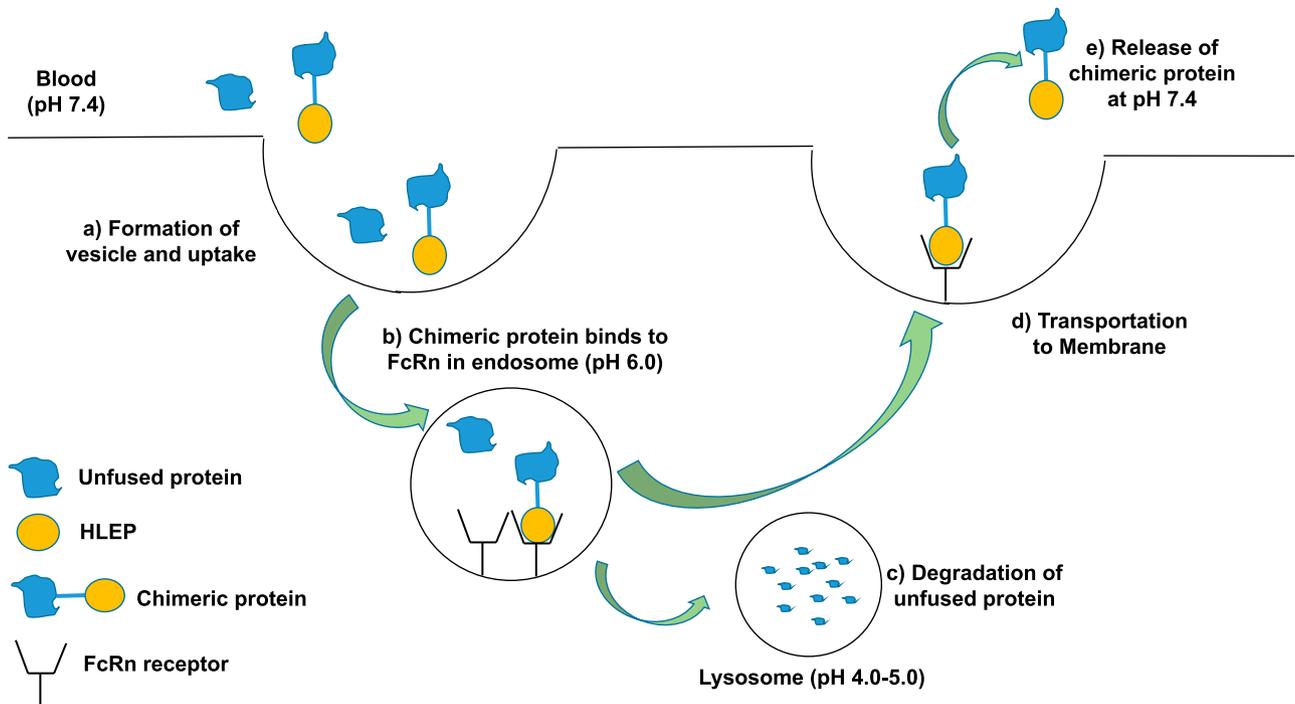
To improve the pharmacokinetic attributes of therapeutic proteins, several approaches have been devised (Fig. 2) (Kontermann, 2011, 2016; Strohl, 2015; Zaman et al., 2019).

### Mechanisms for Improvement in Pharmacokinetics of Protein Therapeutics

One of the most common approaches is to increase the hydrodynamic radius of protein therapeutics, which would lead to reduced renal clearance and increased residence time in the circulation (Kontermann, 2011, 2016; Strohl, 2015). Second approach is to use formulations that entrap the therapeutic proteins, thereby resulting in decreased proteolysis and recognition by phagocytic cells (Hartung and Bendas, 2012). A third approach is to impart negative charge onto the proteins, which would selectively impede their renal clearance (Kontermann, 2011, 2016; Meibohm, 2012; Strohl, 2015). Lastly, attachment or genetic fusion with another protein/domain or recombinant polymeric peptide repeats that have inherently long half-life can be used as an approach to increase the hydrodynamic radius and facilitate FcRn-mediated recycling (Meibohm, 2012; Strohl, 2015; Kontermann, 2016). These approaches of improving pharmacokinetics of therapeutic proteins are discussed in this section.

### Conjugation, Attachment, and Modification

Researchers have devised several methods of chemical conjugation, with one of the classic methods being PEGylation (Kontermann, 2012; Zaman et al., 2019). PEGylation is described as covalent bonding of polyethylene glycol (PEG) moieties with therapeutic proteins (Kontermann, 2012). It has been observed that binding of a few molecules of water to the ethylene glycol subunit results in up to 10-fold increase in size of the PEGylated molecule in comparison with a protein of



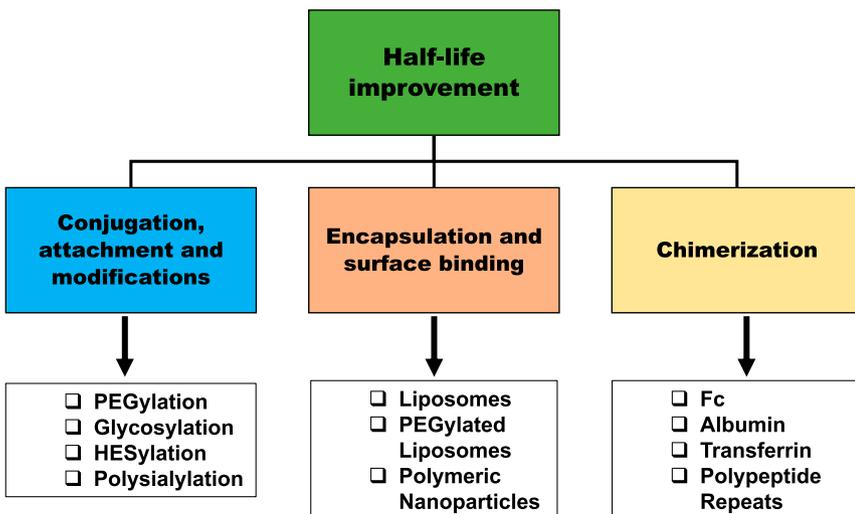
**Fig. 1.** Schematic depiction of FcRn-mediated recycling of chimeric proteins. Proteins chimerized with Fc portion of Ig or albumin are taken up by nonspecific mechanisms into the cell. Once taken up into the cell, the chimeric proteins bind to FcRn at pH 6.0 in the endosome and are recycled back into the circulation, where the chimeric proteins dissociate from the FcRn, as a result of low affinity, due to shift in pH to 7.4. Unfused proteins that do not bind to FcRn are degraded in the lysosome at pH 4.0 to 5.0 (Sokolosky and Szoka, 2015).

similar mass (Swierczewska et al., 2015). Therefore, binding to PEG leads to considerable increase in the size and mass of PEGylated molecule, thereby increasing the hydrodynamic radius and impeding the clearance of PEGylated molecule, consequently resulting in increased half-life (Kontermann, 2012). Furthermore, the PEG conjugation also protects the therapeutic protein from proteolysis and immunologic response (Jevševar and Kunstelj, 2012; Swierczewska et al., 2015). In addition, conjugation with other carbohydrates (e.g., glycosylation, hydroxyethyl starch (HESylation), polysialylation) and synthetic polypeptides (designated as PEPylation) has also gained attention as an alternative to PEGylation (Sinclair and Elliott, 2005; Li and d’Anjou, 2009; Solá and

Griebenow, 2010; Kontermann, 2011, 2012, 2016; Fares, 2012; Vugmeyster et al., 2012; Hou et al., 2019).

**Encapsulation and Surface Binding**

Several pharmaceutical delivery systems, such as liposomes and other polymeric formulations, have also been used to improve the circulatory half-life of therapeutic proteins (Colletier et al., 2002; Hartung and Bendas, 2012; Landfester et al., 2012). Liposomes are bilayered phospholipid vesicles with hydrophilic interior; moreover, PEG modification of liposomes helps surpass clearance by reticuloendothelial system or by phagocytic cells, thereby leading to increased half-life (Hartung and Bendas, 2012). Encapsulation in



**Fig. 2.** Various approaches available to increase circulatory half-life of protein therapeutics.

liposomes has been shown to increase the half-life of tumor necrosis factor- $\alpha$  and interleukin (IL)-2 (Hartung and Bendas, 2012). Another approach to improve pharmacokinetics using liposomes is noncovalent interaction of the protein (e.g., recombinant factor VIII) on the surface of PEGylated liposomes (Hartung and Bendas, 2012). In addition, certain polymeric nanoparticles, nanocapsules, and nanoghosts are also used for pharmacokinetically efficient delivery of peptides and proteins (Pisal et al., 2010; Kontermann, 2012; Landfester et al., 2012; Swed et al., 2014; Krishnamurthy et al., 2019).

### Focus on Chimerization of Protein Therapeutics

**The Concept and Molecular Biology of Chimerization.** The word “chimaera”, also spelled as chimera, holds its origins in the Greek mythology, where chimaera is defined as a monstrous creature of Lycia, which is believed to be a hybrid (<http://www.perseus.tufts.edu/hopper/text?doc=Perseus:text:1999.04.0062:entry=chimaera-harpers>). Chimaera is portrayed as having combination of physical attributes of a lion, a goat, and a snake (<http://www.perseus.tufts.edu/hopper/text?doc=Perseus:text:1999.04.0062:entry=chimaera-harpers>). The concept of chimerism is significant in terms of genetics because it exists in both animal and plant kingdoms; furthermore, experimentally generated chimeras have been serving as useful tools for developmental biologists (Dunsford et al., 1953; Norris et al., 1983; Fontaine-Pérus, 2000; Santelices, 2004; Eckardt et al., 2011). Moreover, certain chimeras, in their incipient stages, could also act as source of organs for transplantation in the future (<https://news.nationalgeographic.com/2017/01/human-pig-hybrid-embryo-chimera-organs-health-science/>).

In terms of protein therapeutics, chimerization is a process where a hybrid is generated with genetic fusion of multiple distinct entities (Baldo, 2015). Hence, in protein chimerization, the idea behind the mythological creature “chimera” is used to produce a molecule with superior properties. A typical protein chimera is produced by connecting genes of the protein molecule of therapeutic interest (“effector”) to another protein/domain (referred to as “helper”) with the help of a “linker”, where the effector molecule possesses a

myriad of pharmacological activities (Czajkowsky et al., 2012; Schmidt, 2013a; Baldo, 2015). The protein chimera or chimerized protein is also referred to as a “fusion protein” or “chimeric protein”, and these terms are used interchangeably (Kontermann, 2012, 2016; Baldo, 2015). The helper imparts stability to the molecule and helps in targeting the effector (Baldo, 2015). Several of these helpers used as half-life extension partners (HLEP) are either full-length proteins or truncated domains of proteins and result in a considerable increase in the half-lives of the effector molecules (Kontermann, 2011, 2016; Strohl, 2015). The purpose of the linker is to connect the effector and helper in a way that allows optimum functionality, so that the whole chimerized molecule can execute its operation (Schmidt, 2013a; Baldo, 2015).

The protein chimerization for half-life extension involves fusing the therapeutic protein with a HLEP, which has inherently longer half-life, e.g., albumin fusion (Fig. 3). (Meibohm, 2012; Strohl, 2015; Sun and Michaels, 2018). Fusing multiple repeats of amino acid sequences (recombinant polymeric peptide repeats) with the effector protein also leads to increased hydrodynamic radius, e.g., elastin-like polypeptides (ELPs) (Strohl, 2015; Kontermann, 2016) (Fig. 3). Using a negatively charged protein fragment that decelerates renal elimination has also been used as HLEP, e.g., C-terminal peptide (CTP) of human chorionic gonadotropin hormone (Meibohm, 2012; Strohl, 2015; Kontermann, 2016; Sun and Michaels, 2018) (Fig. 3).

**Various HLEPs Used to Enhance Half-Life of Protein Therapeutics.** As discussed before, a typical chimeric protein therapeutic is composed of three components: the effector protein is fused to HLEP via a linker peptide. Various partners/helpers used for improving the pharmacokinetics of therapeutic effector proteins are elaborated in this section (Fig. 3).

*Crystallizable fragment of immunoglobulin (Fc).* Fusion with the Fc region of IgG is one of the most popular approaches used to prolong the half-lives of protein therapeutics (Wu and Sun, 2014; Strohl, 2015; Kontermann, 2016; Richter et al., 2019; Zaman et al., 2019). As discussed before, FcRn-mediated recycling plays an important role in recirculation of proteins, particularly in case of fusions containing the Fc region (Fig. 1)

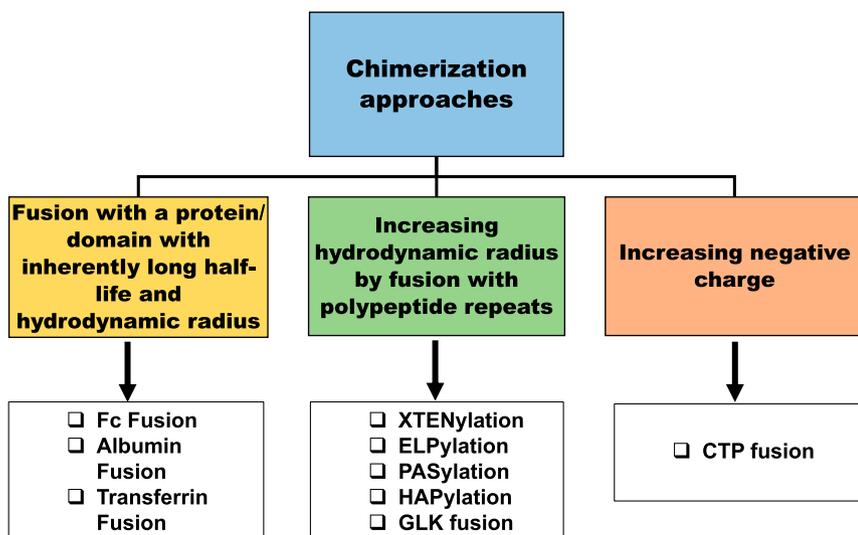


Fig. 3. Various HLEPs available for generation of chimeric protein therapeutics (Strohl, 2015).

(Meibohm, 2012; Rath et al., 2015; Sockolosky and Szoka, 2015; Ward and Ober, 2018). Because the immunoglobulins contain the Fc region, they have substantially long half-lives, e.g., IgG1, IgG2, and IgG4 (Huang, 2009; Meibohm, 2012; Sockolosky and Szoka, 2015; Kontermann, 2016). In case of therapeutic antibodies, half-life extension of as much as 4 weeks has been observed (Keizer et al., 2010; Kontermann, 2016). The function of Fc fusion is to bestow properties such as FcRn-mediated recycling to decrease the metabolism of the therapeutic proteins (Rath et al., 2015; Strohl, 2015; Kontermann, 2016). However, Fc fusions do not possess half-lives as long as the immunoglobulins, which could be in part due to the involvement of fragment antigen binding (Fab) of Ig toward FcRn binding (Suzuki et al., 2010; Schoch et al., 2015; Souders et al., 2015; Kontermann, 2016; Unverdorben et al., 2016). Several undesirable effects are also associated with Fc fusion, such as it may facilitate antibody-dependent cellular cytotoxicity and phagocytosis and complement fixation (Kontermann, 2016). By introducing mutations at specific positions in the Fc region and using particular isotypes of IgG that do not bear such effects, such as IgG4, these undesirable effects can be subsided (Kontermann, 2016). Fc fusion also provides the flexibility of fusing effector molecule(s) at either or both N and C terminus (Kontermann, 2016). Several Fc fusions are developed and approved by various drug regulatory authorities, including etanercept (tumor necrosis factor receptor fusion), aflibercept (vascular endothelial growth factor receptor fusion), and rilonecept (IL-1 receptor fusion) (Jazayeri and Carroll, 2008; Huang, 2009; Strohl, 2015; Kontermann, 2016). Mutations in the Fc regions have been performed for optimizing the properties of the resultant fusions and to overcome undesirable effects. For example, dulaglutide, a fusion of GLP-1 with Fc region of IgG4, is a GLP-1 receptor agonist with mutations F234A and L235A (Kontermann, 2016). These mutations decrease Fc $\gamma$  receptor interaction and antibody-dependent cellular cytotoxicity induction (Kontermann, 2016). The mutation S228P in dulaglutide prevents the formation of half-antibodies (Glaesner et al., 2010; Kontermann, 2016). When compared with other GLP-1 agonists, such as exenatide and liraglutide, which require daily administration, dulaglutide has to be applied only once in a week (Kontermann, 2016). Mutations that cause an upsurge in FcRn binding, thereby increasing the half-life, can also be incorporated in the Fc region (Presta, 2008; Kuo and Aveson, 2011; Wang et al., 2014b; Kontermann, 2016). Several mutations that have led to considerable half-life improvements of IgGs are reported in the literature (Presta, 2008; Kuo and Aveson, 2011; Wang et al., 2014b; Kontermann, 2016; Bas et al., 2019). For example, motavizumab, which is a monoclonal antibody for respiratory syncytial virus, incorporates three mutations in the Fc region, namely M252Y, S254T, and T256E (Robbie et al., 2013; Kontermann, 2016). These mutations increase the FcRn binding up to 10-fold and the half-life in serum from 2- to 4-fold (Robbie et al., 2013; Kontermann, 2016; Liu, 2018). Moreover, it has been reported that hypersialylation of asparagine residue at 297 position, achieved through deletion of glutamate residue at 294, could prolong the residence of Fc in the serum (Bas et al., 2019). Also, fusion with certain FcRn-binding peptides has led to enhancement in the half-life of Fab domain (Datta-Mannan et al., 2019). Apart from direct Fc chimerization, fusion with IgG-binding domain of streptococcal G protein, which

possesses binding capability toward Fc, has also been used for half-life extension (Unverdorben et al., 2015; Zong et al., 2019). Eftrenonacog- $\alpha$ , marketed as Alprolix, which is a Fc-fused factor IX for treatment of haemophilia, is an example of recently approved Fc fusion therapeutic (Table 1) (Shapiro et al., 2012, 2018; Strohl, 2015; Graf, 2018). In addition, Eloctate (Fc fused factor VIII) has also been studied clinically for the treatment of haemophilia (Mahlangu et al., 2018). Studies with Fc-fused cocaine hydrolase for the treatment of cocaine abuse and fusion of growth hormone with single-chain Fc dimer have also been performed (Zhou et al., 2017; Chen et al., 2018).

*Albumin.* It has been observed that the half-life of albumin, a predominantly abundant protein in the serum, is up to 19 days (Sleep et al., 2013; Sleep, 2015; Kontermann, 2016). FcRn-mediated recycling also plays a pivotal role in preventing albumin catabolism, but because albumin binds to FcRn at a site different from IgGs, there is no interference with the recycling of IgGs (Sand et al., 2015; Kontermann, 2016; Larsen et al., 2018). The first and the third domain of albumin are found to interact with FcRn in a pH-dependent manner (Sand et al., 2014). Because albumin acts as a transport protein, it can be used for half-life improvement of protein therapeutics (Sleep, 2015; Kontermann, 2016). Half-life extension approaches using albumin involve either binding to albumin, via various interactions and conjugations, fusion of target proteins to albumin-binding moieties, or by generating chimeras with direct albumin genetic fusion (Kontermann, 2016; Larsen et al., 2016; Lee and Youn, 2016; Fuchs and Igney, 2017; Ramírez-Andersen et al., 2018; Baghban Taraghdari et al., 2019; Zaman et al., 2019). Albumin-binding moieties include certain fragments of antibodies (such as Fab), single-chain variable fragment (scFv), domain antibodies, nanobodies, albumin-binding domains, DARPin domains, and albumin-binding peptides (Tijink et al., 2008; Rycroft and Holt, 2012; Schmidt et al., 2013; Sleep et al., 2013; Goodall et al., 2015; Jacobs et al., 2015; Sleep, 2015; Van Roy et al., 2015; Kontermann, 2016; Li et al., 2016; Steiner et al., 2017; Khodabakhsh et al., 2018; Seijsing et al., 2018; Ikeda et al., 2019; Jank et al., 2019). When albumin-binding moieties, attached to therapeutic proteins by genetic fusion, are administered, they bind to serum albumin, thereby increasing the half-life of fused therapeutic (Kontermann, 2016). However, direct fusion with albumin is one of the significant approaches for half-life extension and has been used for a wide variety of molecules with therapeutic properties (Müller et al., 2007; Strohl, 2015; Kontermann, 2016; Hoogenboezem and Duvall, 2018). One notable example is the fusion of human serum albumin with GLP-1, referred to as albiglutide, has a half-life ranging from 4 to 7 days, and has been approved for diabetes treatment (Table 1) (Bush et al., 2009; Trujillo and Nuffer, 2014; Strohl, 2015). Another example is the fusion of albumin to factor IX, referred to as albutrepenonacog alfa and marketed as Idelvion for haemophilia treatment (Santagostino et al., 2016; Chia et al., 2018; Graf, 2018). Moreover, other clotting factors such as factor VIIa, VIII, and X have also been fused with albumin (Schulte, 2009; Tiede, 2015; Kontermann, 2016; Ferrarese et al., 2019). Recently, fusion of albumin with glucaripidase and Kunitz protease inhibitor domain of protease nexin 2 has also been investigated (Sheffield et al., 2018; AlQahtani et al., 2019). Derivatives of albumin with enhanced properties can be produced by introducing mutations

TABLE 1

Examples of protein partners used in the generation of chimeric protein therapeutics with improved half-life

The NCT number represents the clinicaltrials.gov identifier for the clinical study of the respective drug molecule. Web addresses for information taken from clinicaltrials.gov (accessed 02-04-2019): <https://clinicaltrials.gov/ct2/show/NCT02526420>; <https://clinicaltrials.gov/ct2/show/NCT02339090>; <https://clinicaltrials.gov/ct2/show/NCT01658501>; <https://clinicaltrials.gov/ct2/show/NCT02968004>.

Help	Effector Protein (Drug Name)	Half-Life with and without Chimerization		Disease Indication	Development Status	References
		Without	With			
Fc fusion	Factor IX (eftronacog- $\alpha$ )	~18 h	~57 h	Haemophilia	Approved by FDA in 2014	Shapiro et al. (2012), Strohl (2015)
Albumin fusion	GLP-1 (albiglutide)	1–2 min	~4–7 day	Diabetes mellitus	Approved by FDA in 2014	Strohl (2015), Kontermann (2016)
Tf fusion	Proinsulin	0.5 h	>7 h	Diabetes mellitus	Preclinical (BALB/c mice)	Wang et al. (2014b)
XTENylation	Growth hormone (GH) (VRS-317/somavaratan)	1.7 h	131 h (in adults)	GH deficiency	Phase 2 for adults (NCT02526420) Phase 3 for children (NCT02339090)	Fares et al. (2010), Yuen et al. (2013), Strohl (2015), Moore et al. (2016)
PASylation	Adenectin	49 min	226 min	Cancer	Preclinical (BALB/c mice)	Aghaabdollahian et al. (2019)
ELPylation	GLP-1 (PB1023)	1–2 min	~36 h	Diabetes mellitus	Phase 2 (NCT01658501)	Strohl (2015)
CTP fusion	Growth hormone (GH) (MOD-4023)	1.7 h	~33–37 h	GH deficiency	Phase 3 (NCT02968004)	Fares et al. (2010), Hershkovitz et al. (2016), Strasburger et al. (2017)

FDA, Food and Drug Administration.

at certain positions, such as a mutation K573P results in enhanced FcRn affinity by up to 11-fold, thereby leading to increased half-life (Andersen et al., 2014; Kontermann, 2016). In addition, fusion to albumin can be performed at both N or C terminus or individually at either N or C terminus (Müller et al., 2007; McDonagh et al., 2012; Andersen et al., 2014; Rogers et al., 2015; Strohl, 2015; Kontermann, 2016).

**Transferrin.** Transferrin (Tf), which is a highly abundant monomeric glycoprotein of 80 kDa and takes part in uptake of iron in the cells by exocytosis and receptor-mediated endocytosis (through Tf receptor), is also used as HLEP (Li and Qian, 2002; Chen et al., 2013a; Strohl, 2015; Kontermann, 2016). It is found to have a half-life of up to 17 days; however, the glycosylated counterpart of Tf may have a reduced half-life of up to 10 days, and hence the nonglycosylated Tf is preferred as a fusion partner (Kim et al., 2010; Strohl, 2015; Kontermann, 2016). The prominent example of Tf fusion is the fusion of GLP-1 agonist and exendin-4 with nonglycosylated Tf (Kim et al., 2010). Fusions of GLP-1 with Tf have shown a half-life of 44 hours in cynomolgus monkey model and resulted in decrease in blood glucose and increase in insulin secretion (Kim et al., 2010; Kontermann, 2016). In another study, the Tf–proinsulin fusion displayed a substantial increment in the elimination of half-life of 15-folds in comparison with the half-life of unfused proinsulin (Table 1) (Wang et al., 2014a). Moreover, a recent study with Tf–proinsulin fusion demonstrates that the fusion possessed a lowering effect on blood glucose levels for up to 40 hours (Shao et al., 2016). Other examples include development of Tf fusions with growth hormone and granulocyte colony-stimulating factor (Chen et al., 2011; Kontermann, 2016).

**Fusion with other half-life extension partners.** As a substitute to PEGylation, certain approaches have been developed that use recombinant fusion of target protein with recombinant polymeric peptide repeats that are also termed as PEG mimetics (Kontermann, 2012, 2016; Strohl, 2015; Sun and Michaels, 2018). Fusion to these recombinant polypeptide chains leads to increase in the hydrodynamic radius of the chimeric proteins, thereby causing impeded renal elimination (Strohl, 2015; Kontermann, 2016). Several approaches for chimerization of therapeutic proteins using these recombinant polypeptide repeats are described in this section. XTENylation is a half-life extension approach developed by Amunix, which utilizes fusion of polymeric amino acid sequences containing alanine, glutamic acid, glycine, proline, serine and threonine, designated as XTEN, to the target protein (Schellenberger et al., 2009; Strohl, 2015). XTEN polymer of 864 amino acids in length has been shown to extend half-lives of several therapeutically important proteins both in the form of fusion and chemical conjugation (Schellenberger et al., 2009; Alters et al., 2012; Podust et al., 2013, 2016; Strohl, 2015). One of the examples for XTEN fusions is VRS-317 (somavaratan), a fusion of XTEN and human growth hormone (Table 1) (Cleveland et al., 2012; Yuen et al., 2013; Strohl, 2015; Moore et al., 2016). Another example of polypeptide repeats used for half-life extension is ELPylation, which utilizes peptide polymer repeats that are generally found in hydrophobic domain of Elastin, hence the term, elastin like polypeptides (ELPs) (Hassounah et al., 2012; MacEwan and Chilkoti, 2014; Strohl, 2015). ELPs are formed with combination of five peptides, valine-proline-glycine-x-glycine, where x could be any guest amino acid apart from proline (Floss et al., 2010,

2013; Hassouneh et al., 2012; MacEwan and Chilkoti, 2014; Strohl, 2015). ELPs are biodegradable owing to their metabolism by the elastases in the body (Strohl, 2015). ELPylation impedes kidney clearance by increasing the hydrodynamic radius, thereby enhancing the half-life of protein therapeutics (Conrad et al., 2011; Strohl, 2015). Some examples of ELPylated fusions include Glymera (PB1023), which is a GLP-1 fusion, and Vasomera, a fusion with vasoactive intestinal peptide (Table 1) (Strohl, 2015). Recently, a study has shown that ELPylation of interferon- $\alpha$  led to the formation of sustained release depot that significantly prolonged the action of interferon- $\alpha$  for antitumor activity (Wang et al., 2019). PASylation is another approach toward half-life extension of therapeutic proteins, in which recombinant polymeric repeats are formed by using proline, alanine, and serine amino acids (therefore termed as PASylation) (Schlupschy et al., 2013; Binder and Skerra, 2017; Breibeck and Skerra, 2018; Gebauer and Skerra, 2018). Several studies for half-life extension using PASylation are reported in the literature, one of the notable examples being the PASylation of exenatide, in which a PAS repeat of 600 amino acids led up to 100-folds increment in the half-life (Schlupschy et al., 2013; Harari et al., 2014; Strohl, 2015; Gebauer and Skerra, 2018). A recent example for PASylation is the fusion of Adnectin C with PAS repeat of 200 amino acids, which led to increase in half-life by a factor of 4.5 (Table 1) (Aghaabdollahian et al., 2019). In addition to the above-mentioned approaches for half-life extension, some others include HAPylation, which utilizes homo-amino-acid polymers (HAPs) that consist of glycine-rich repeat sequences, and gelatin-like protein fusion (Schlupschy et al., 2007; Kontermann, 2009; Huang et al., 2010; Strohl, 2015). Apart from using polypeptide repeats, fusions of therapeutic proteins with CTP of the  $\beta$ -subunit of human chorionic gonadotrophic hormone have also been generated (Fares et al., 1992; Calo et al., 2015; Fares and Azzam, 2019). This imparts or increases the negative charge on the chimeric protein, thereby impeding renal elimination (Fares et al., 1992; Calo et al., 2015). Some recent examples of CTP fusion include MOD-4023, which is a fusion with human growth hormone, and MOD-5014, which is CTP fusion with factor VIIa (Table 1) (Hershkovitz et al., 2016; Strasburger et al., 2017; Bar-Ilan et al., 2018).

*Linkers: the bond between the effector and helper.* The selection or rational design of a linker to join the effector and HLEP protein is a critical area in chimeric protein technology (Kontermann, 2011, 2016; Chen et al., 2013a,b). The linker peptide helps to connect the protein components and could also have a pivotal role in interdomain/interprotein interactions and in preserving the respective biologic activity

(Gokhale and Khosla, 2000). Moreover, linkers can also have positive effects on the stability, activity, and pharmacokinetics of chimeric proteins (Chen et al., 2013b). Direct fusion of proteins to generate a chimera without any linker may lead to unwanted effects, such as low yield and reduced activity (Bai et al., 2005; Bai and Shen., 2006; Zhao et al., 2008; Amet et al., 2009; Chen et al., 2013b). Thus, choice and design of linkers are very important. Based on their attributes, linkers can be classified as flexible, rigid, and cleavable (Table 2) (Chen et al., 2013a,b). Flexible linkers are used when the protein partners/domains in the chimera require movement, interaction, and maintenance of a certain distance between them. They are composed of small, nonpolar, or polar amino acids. The polar amino acids facilitate hydrogen bonding, and the small size of amino acids helps to achieve flexibility (Argos, 1990; Chen et al., 2013a,b). The most commonly used flexible linker is the GS linker with sequences consisting of glycine and serine residues, and the sequence formula (Gly-Gly-Gly-Gly-Ser) $_n$  or (G $_4$ S) $_n$  is the most popular among flexible linkers, where  $n$  is the number of repeats (Chen et al., 2013a,b) (Table 2). Other examples of flexible linkers include (Gly) $_8$  or (G) $_8$  linker and the linkers used to create scFv such as KESGSVS and EGKSSGSGSESKST (Bird et al., 1988; Sabourin et al., 2007; Chen et al., 2013a,b). The flexible linkers, however, owing to their high flexibility, might not allow the separation of the operational domains/protein partners (Chen et al., 2013b). Moreover, it has been reported that the use of flexible linker may result in failed expression of certain chimeric constructs (Bai and Shen, 2006; Amet et al., 2009; Chen et al., 2013b).

Therefore, where a spatial separation of domains/protein partners is required, rigid linkers are used because they act as inflexible spacer peptides that separate domains/protein partners (Table 2). Due to this inflexible distance, the domains/protein partners are relatively free to perform their respective functions (Amet et al., 2009; Chen et al., 2013a,b). Two types of rigid linkers are predominantly mentioned in the literature. The first is the  $\alpha$ -helical linker with the formula (EAAAK) $_n$ , where  $n$  is the number of repeats; the second is the rigid (XP) $_n$  linker, where  $n$  is number of repeats and P is proline; here X can be any amino acid; however, generally alanine, lysine, and glutamine are used (Table 2) (Chen et al., 2013a,b). Due to the inability of proline to form hydrogen bonds, the domain-linker/protein-linker interaction is avoided, and this increases the rigidity leading to efficient separation of protein partners in the chimera (Chen et al., 2013a,b). The first two categories of linkers contain noncleavable and stable sequence of peptides that can impart several advantages, including conformational

TABLE 2

Various linkers used for chimerization of proteins (Chen et al., 2013a,b)

Type of Linker	Properties	Example
Flexible linkers	Composed of small polar/nonpolar amino acids. Used when domains require movement.	(G $_4$ S) $_n$ , (G) $_8$
Rigid linkers	Do not allow separation of operational domains/protein partners. Used when spatial distance between domains/protein partners is required.	(EAAAK) $_n$ , (XP) $_n$
Cleavable linkers	Freedom for domains to perform respective functions. Free functional protein partners released subsequent to cleavage in vivo. Cleaved in the presence of proteases or reducing agents.	VSQTSKLTRAETVFPDV, dithiocyclopeptide linker

flexibility, improved stability, and activity (Chen et al., 2013a,b). However, with the use of these stable peptide linkers, several detrimental effects, such as decrements in activity and steric hindrances in domains/protein partners, have been observed (Chen et al., 2013a,b). To overcome this, a third category of the cleavable linkers is used with the intention of releasing free functional domains/protein partners in vivo (Chen et al., 2010, 2013a,b) (Table 2). These linkers are cleaved under some specific conditions such as presence of reducing reagents or proteases. The first type in this category is the in vivo cleavable disulfide linker. One of the examples of this is the dithiocyclopeptide linker, which is cleaved in presence of a reducing environment (Chen et al., 2013a,b) (Table 2). The second type are the in vivo protease-sensitive linkers in which the chimera is designed with a linker containing a protease-sensitive sequence (Chen et al., 2013a,b). An example of this is a fusion between recombinant factor IX and albumin, in which a sequence, VSQTSKLTRAETVFPDV, from N-terminal region of factor IX, which is susceptible to proteolytic cleavage, is incorporated (Table 2) (Schulte, 2009; Chen et al., 2013a,b). This causes several fold increments in the clotting activity of the chimeric protein as compared with the chimeric protein with noncleavable linkers (Schulte, 2009; Chen et al., 2013b).

### Protein Chimerization for Miscellaneous Specialized Purposes

The choice of helper protein is primarily based on the desired functions of the chimeric protein because the helper partners (apart from half-life extension) can also be used to generate chimeras with other properties such as cancer targeting and permeation through blood brain barrier (Tijink et al., 2008; Pardridge, 2015; Hoogenboezem and Duvall, 2018; Jank et al., 2019). For instance, human paraoxanase-1 fused with the C terminus of heavy chain of monoclonal antibody against human insulin receptor has the ability to cross blood brain barrier (Boado et al., 2008). In addition, NHS-IL2, a chimera of IL-2 and NHS76 (an IgG1 targeting tumor necrosis), not only displayed half-life extension, but also showed selectivity in targeting tumors in mice (Fallon et al., 2014). In addition, an antibody developed with fusion of scFv with Fc showed significant in vitro growth inhibition of *Staphylococcus aureus* (Wang et al., 2019).

Chimerization with HLEPs could also have synergistic effect on the pharmacological activity of the effector, e.g., Fc fusions with Osteopontin and Follistatin-288 have shown to enhance the osteogenic effect and promote localized growth of skeletal muscles, respectively (Castonguay et al., 2019; Rattanapisit et al., 2019). In addition, fusion of canine interferon- $\gamma$  with canine serum albumin not only led to improved pharmacokinetics, but also improved antitumor efficacy (Li et al., 2019). Similarly, fusion of recombinant immunotoxins with albumin-binding domains led to increased half-life and significant increment in the antitumor effects of these immunotoxins (Wei et al., 2018). Thus, effector proteins with different functions can be chimerized together to produce a multifunctional protein therapeutic (Jochems et al., 2018).

### Comparison and Outlook on Shortcomings of Protein Chimerization versus Other Approaches Used for Improvement of Pharmacokinetics

Various approaches of half-life extension have their respective shortcomings. PEGylation, for instance, has many disadvantages that include reduced activity of the conjugated protein, increased aggregation, and unusual behavior of the therapeutic, such as increase in activity in some cases (Wang et al., 2007; Veronese and Mero, 2008; Pisal et al., 2010; Kontermann, 2016). Because PEG is nondegradable in circulation, it may lead to renal, hepatic, and splenic vacuolization (Pelegri-O'Day et al., 2014; Zhang et al., 2014; Qi and Chilkoti, 2015). Immunologic response subsequent to administration of PEGylated molecules is another prevailing issue (Swierczewska et al., 2015; Kontermann, 2016). Development of antibodies against PEGylated therapeutic products such as Krystexxa (PEGylated Uricase) and Oncospar (PEGylated Asparaginase) has been reported and may lead to accelerated clearance of the therapeutic (Armstrong, et al., 2007; Sundry et al., 2011; Garay et al., 2012; Swierczewska et al., 2015). In case of glycosylation, the performance of the therapeutic protein may be affected by aberrant glycosylation patterns that can further lead to rapid clearance through mannose and asialoglycoprotein receptors, and, in addition, certain carbohydrate structures may lead to development of an immune response (Jenkins and Curling, 1994; Jenkins et al., 1996; Vugmeyster et al., 2012). In case of pharmaceutical formulation such as liposomes, their stability in blood is questionable due to low critical micelle concentration (Landfester et al., 2012).

One of the important issues faced by protein therapeutics is the formulational stability of the finished product (Strohl, 2015). The critical aspect of formulation stability lies in poor solubility and tendency of chimeric proteins to form aggregates and micelles; however, the introduction of glycosylation sites in the chimera might help in overcoming aggregation (Huang and Swanson, 2013; Strohl, 2015). In addition, immunogenicity is the bottleneck for the application of therapeutic proteins (De Groot and Scott, 2007; Jawa et al., 2013; Baldo, 2015; Kimchi-Sarfaty et al., 2017). It is crucial that the chimeric protein must elicit negligible to very low immune response, subsequent to administration; this is particularly important for chimeras developed for long-term therapy (Strohl, 2015; Swierczewska et al., 2015). Proteins are recognized by the immune system owing to the presence of T and B cell epitopes; in case of chimerized proteins, immunogenic response against the protein of interest could potentially worsen the disease condition in patients (Purcell and Lockey, 2008; Strohl, 2015). In addition, chimerization of proteins may lead to the formation of new epitopes that may elicit further immunogenic response (Strohl, 2015). Furthermore, several aspects relating to molecular structure and formulation affect the immunogenicity of therapeutic proteins (Purcell and Lockey, 2008; Strohl and Strohl, 2012; Schmidt, 2013; Strohl, 2015). However, there have been lesser incidences of immunogenic response and anti-drug antibody formation subsequent to administration of chimeric proteins (Strohl, 2015). One of the methods used to reduce immunogenicity includes the prediction and elimination of T cell epitopes in a chimeric protein (Strohl, 2015). In addition, stability of finished product (such as optimal solubility and lack of

aggregation) also helps in eliminating the development of immunogenic response (Strohl and Strohl, 2012; Jawa et al., 2013; Baldo, 2015; Strohl, 2015).

In case of Fc fusions, the issue of inconsistent glycosylation either in linker or in chimera and the functionality of Fc domain needs attention (Strohl, 2015). Orientation of the effector molecule may also play a key role, as the binding of effector toward N or C terminus of the HLEP may significantly affect its activity (Schmidt, 2013a). Hence, an attempt for fusion of target proteins should be made at both N and C terminus to design chimera with maximum activity. In addition, because linkers also affect the activity and utility of chimeric proteins, the choice of linkers (rigid, flexible, and cleavable) should be made as per the desired therapeutic effect of the target protein (Chen et al., 2013b; Schmidt, 2013a). Furthermore, research should also be focused toward exploring the different avenues of linker design, to provide much wider variety and combinations.

Studies done on recombinant polymeric peptide repeats have been limited in contrast to Fc, HSA, and transferrin fusion (Strohl, 2015). Therefore, much is unknown about these approaches, and elaborate studies are required to establish them as candidates for HLEP, and because these platforms are unnatural repeats of amino acids, use of these approaches may also raise several multifaceted issues (Strohl, 2015). Approaches such as HAPylation and gelatin-like protein fusion offer small half-life improvements, and mostly are in their incipient stages, and much study is required to establish a clinical basis for the use of these approaches (Strohl, 2015). In case of CTP fusion, owing to the strong negative charge, the biologic activity of the chimera may be affected (Strohl, 2015). However, considering the half-life extension afforded by the fusion with recombinant polymeric peptide repeats and CTP, their use seems to be more beneficial than approaches involving conjugation and encapsulation (Strohl, 2015; Hershkovitz et al., 2016; Strasburger et al., 2017; Bar-Ilan et al., 2018; Gebauer and Skerra, 2018).

Therefore, considering all of the currently known facts, it seems that the protein chimerization is more advantageous in comparison with other approaches for half-life extension (e.g., PEGylation, glycosylation, liposome formulation) for delivery of protein therapeutics.

## Conclusion and Future Prospects

Biopharmaceuticals are clearly leading the way for pharmaceutical therapy. The number of approvals from January 2015 to July 2018 (~3.5 years) was almost double in comparison with approvals in each 5-year span from 1995 to 2014 (Walsh, 2018). Interestingly, among the products approved between January 2014 and July 2018, >90% were protein therapeutics that include monoclonal antibodies, clotting factors, enzymes, and vaccines (Walsh, 2018). This suggests that protein therapeutics have become the cornerstone of biologic therapy. Furthermore, since their inception, half-life extension technologies have come very far, and with the development of newer approaches such as Fc fusion, albumin fusion, Tf fusion, etc., we now have a wide variety of approaches to choose from (Kontermann, 2012, 2016; Strohl, 2015). However, even with such remarkable advancements, there are considerable challenges that need to be addressed.

With the increasing discovery of novel pathophysiological mechanisms of various diseases, the significance of protein therapeutics in the current scenario for disease interventions is more than ever before. However, the application of an emerging protein therapeutic may be hindered due to its poor pharmacokinetic attributes. This clearly emphasizes that mere discovery may not be enough for most protein therapeutics; engineering them for optimum pharmacokinetics and pharmacodynamics is equally important. Furthermore, creation of computational methods, programs, or software applications for the design and engineering of the chimera, with integrated systems for the prediction of immunogenicity, can ease out and accelerate the development of chimeric proteins with improved pharmacokinetic and pharmacodynamic properties (Paladino et al., 2017; Wang et al., 2018). It is important to note that programs for designing linkers for chimeric proteins are already available, e.g., LINKER and SynLinker (Crauto and Feng, 2000; Liu et al., 2015). Such advancements in computational approaches may also lead to the development of tailored protein therapeutics with customizable half-life, which may even have possible applications in personalized medicine. Finally, the development of multifunctional half-life extended chimeric proteins (with multiple effector proteins) for complex disease interventions must be explored in much detail (Chen et al., 2011; Jochems et al., 2018).

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## Authorship Contributions

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